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EFFECT OF BIALAPHOS AND PHOSPHINOTHRICIN ON PLANT REGENERATION FROM LONG- AND SHORT-TERM CALLUS CULTURES OF *GLADIOLUS*

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SUMMARY

Callus was initiated from *in vitro*-grown plants of *Gladiolus* cultivars 'Jenny Lee' and 'Florida Flame.' The age of callus used for regeneration of plants was either 9 mo. old or 8 yr old from 'Jenny Lee,' and 4 mo. old from 'Florida Flame.' Regeneration medium consisted of Murashige and Skoog's basal salts medium supplemented with 2.0 mg/l (9.3 μ*M*) kinetin. This medium was supplemented with various concentrations of either bialaphos (Meiji Seika, Tokyo, Japan) or phosphinothricin (Hoechst-Roussell, Frankfurt, Germany). Bialaphos was more effective than phosphinothricin at stimulating plant regeneration. Plants regenerated from 8-yr-old callus of 'Jenny Lee' only when the regeneration medium was supplemented with 0.10 mg/l bialaphos. A bialaphos concentration of 0.01 mg/l stimulated regeneration from 9-mo.-old callus of cultivar 'Jenny Lee' and 4-mo.-old callus of 'Florida Flame.'

Key words: Gladiolus; callus; phosphinothricin; bialaphos; plant regeneration; long-term callus.

INTRODUCTION

Plants have been regenerated from callus of *Gladiolus* by several researchers (Ziv et al., 1970; Simonsen and Hildebrandt, 1971; Bajaj et al., 1982, 1992; Kim et al., 1988; Kamo et al., 1990; Kamo, 1994, 1995; Stefaniak, 1994; Remotti, 1995; Remotti and Loffler, 1995). In these reports, regeneration of plants was achieved from short-term callus cultures that had been in culture less than 1 yr. A dramatic reduction in the ability to regenerate plants from 10-mo.-old callus has been documented (Kim et al., 1988; Kamo, 1994). Another factor affecting the regeneration capacity of *Gladiolus* callus is the difference in response between cultivars (Stefaniak, 1994; Kamo et al., 1995; Remotti and Loffler, 1995). The regeneration capacity of cells used for transformation greatly affects the frequency of transgenic plants recovered. *Gladiolus* has been transformed by particle gun bombardment of callus and suspension cells (Kamo et al., 1995). Several months were required to multiply the callus and establish suspension cells in amounts needed for particle gun bombardment. Also, after suspension cells were bombarded, they were grown on a selective agent for 3–6 mo. The regeneration capacity of the callus decreases during the time the cells are cultured resulting in a lower transformation efficiency.

A nonselective herbicide, phosphinothricin (PPT), has been used as a selective agent in plant transformations and has been effective in the transformation of several monocots. PPT (trade name Basta by Hoechst AG, Frankfurt, Germany) is a phosphinic acid analogue of L-glutamic acid that inhibits glutamine synthetase (Krieg et al., 1990; Schulz et al., 1990). Bialaphos (trade name Herbiace by Meiji Seika Kaisha, Tokyo, Japan) is a tripeptide consisting of PPT and two L-alanine residues. Bialaphos is a natural product produced by fermentation of *Streptomyces hygroscopicus* as compared to PPT, which is chemically synthesized. The application of both PPT and bialaphos has been shown to result in the rapid accumulation of free ammonia in higher plants. It is not known if ammonia accumulation is the cause of cell death in plants, but results from two studies suggest that this is not the case (Krieg et al., 1990; Palmer and Oelck, 1992).

It has been reported that PPT completely inhibited embryogenesis in alfalfa callus (Krieg et al., 1990). In comparison, this study showed the stimulatory effect of bialaphos on regeneration of plants from callus of *Gladiolus*, making bialaphos a particularly useful agent for selection in transformation research.

MATERIALS AND METHODS

Plant stocks. Plants of *Gladiolus* cultivar 'Jenny Lee' were from Oglesby Plant Laboratories (Altha, FL) and 'Florida Flame' were from the Manatee Fruit Company (Palmetto, FL). Plants were maintained *in vitro* by monthly transfer to Murashige and Skoog's (1962) (MS) basal salts medium (Logan and Zettler, 1985), and they were grown at 25° C under a 16-h light photoperiod (75 μE $m^{-2}s^{-1}$).

Callus cultures. Callus was initiated from *in vitro*-grown plants cultured on MS basal salts medium supplemented with 3% (wt/vol) sucrose, 0.2% (wt/vol) Gelrite (Merck & Company, Fiskeville, RI), 2 mg/l glycine, 1 mg/l thiamine, 0.5 mg/l pyridoxine, 0.5 mg/l nicotinic acid, 100 mg/l myo-inositol, and 0.5 mg/l (2.2 μ*M*) 2,4 dichlorophenoxyacetic acid (2,4-D). The medium was adjusted to pH 5.8 before autoclaving at 121° C, 20 psi for 20 min.

In vitro-grown plants used for callus initiation were grown at 25° C under a 16-h light photoperiod for approximately 4 mo. at which time the callus was removed from the explant. Callus was multiplied by culturing it on the same medium as used for callus initiation. The 8-yr-old callus from cultivar 'Jenny Lee' has been maintained on MS medium supplemented with 2 mg/l 2,4-D to maintain a friable characteristic. Callus cultures were transferred monthly to fresh medium and grown in the dark at 25° C.

Suspension cell cultures. Suspension cultures were initiated from callus cultures (Kamo et al., 1990). Suspension cells were grown in the same medium as used for callus initiation and maintenance, except that Gelrite was

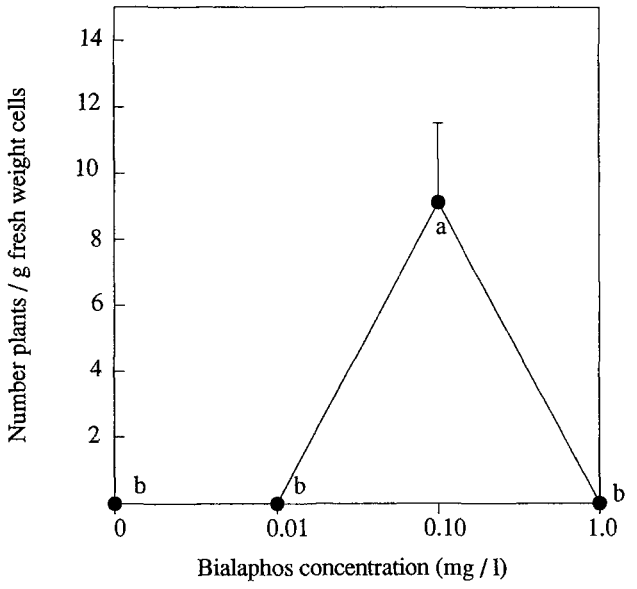

FIG. 1. Regeneration of plants from 8-yr-old callus of 'Jenny Lee' cultured on regeneration medium supplemented with various concentrations of bialaphos. *Standard error bars* shown. Means with different letters are significantly different at $P \leq 0.01$ according to Dunnett's test.

omitted. Suspension cells were transferred weekly to 20 ml fresh medium at a 1:2 dilution and maintained in the dark at 25° C on a gyratory shaker at 120 rpm.

Plant regeneration. Two-mo.-old suspension cultures were used for regeneration studies. Approximately 1 g fresh weight (FW) of suspension cells were placed on a Whatman no. 1 filter paper over solidified regeneration medium consisting of MS basal salts supplemented with 2 mg/l kinetin. For regeneration experiments, either bialaphos or PPT at 0, 0.01, 0.10, or 1.0 mg/l were added by filter sterilization to the regeneration medium. The suspension cells from several flasks were combined so that each replicate came from a single flask of cells. There were four regeneration plates per herbicide concentration for each replicate, and each herbicide concentration series was repeated three times. This was done for each cultivar and age of callus. Plants were counted for regeneration when they were 2-cm tall. All regeneration cultures were transferred once a month for a total of 6 mo. to fresh medium and grown at 25° C under a 16-h light photoperiod.

RESULTS AND DISCUSSION

The various cell lines were selected for regeneration experiments because they differ in their regeneration capacity when cultured on MS basal salts supplemented with 2.0 mg/l kinetin. Cells that had been maintained as callus cultures of 'Jenny Lee' for 9 mo. regenerated plants, whereas 8-yr-old callus of 'Jenny Lee' did not when cultured on regeneration medium. The cultivar 'Florida Flame' did not regenerate plants as readily as 'Jenny Lee' (Kamo, 1995).

Bialaphos stimulated the regeneration of plants from 8-yr-old cells of 'Jenny Lee' (Fig. 1). The effect of 0.10 mg/l bialaphos was statistically significant, and the plants regenerated formed roots and cormels. No plants were regenerated from 8-yr-old callus cultured on regeneration medium supplemented with 0.01 or 0.10 mg/l PPT, and a single plant was observed on one of the 12 plates containing 1.0 mg/l PPT (data not shown). Eight-yr-old callus of 'Jenny Lee' was maintained on MS basal salts supplemented with 2.0 mg/l 2,4-D, and proliferated rapidly. The friable callus has a rough appearance on its surface and was watery. The appearance of the callus changed to resemble embryogenic callus that is hard and white, with a smooth, contoured surface following culture on regeneration medium supplemented with 0.10 mg/l bialaphos (Fig. 4 *A*). Eight-yr-old callus was cultured 4–6 mo. on regeneration medium supplemented with 0.10 mg/l bialaphos before intact plants were regenerated. This same concentration of bialaphos (0.10 mg/l) added to regeneration medium has been used in the successful recovery of transgenic *Gladiolus* plants following particle gun bombardment (Kamo et al., 1995).

The regeneration of plants from 9-mo.-old cells of 'Jenny Lee' was significantly stimulated by 0.01 mg/l bialaphos added to the regeneration medium (Fig. 2 *A*). Regeneration medium supplemented with PPT (0.10 mg/l) also significantly stimulated regeneration of plants in contrast to 8-yr-old cells of 'Jenny Lee,' which were unaffected by

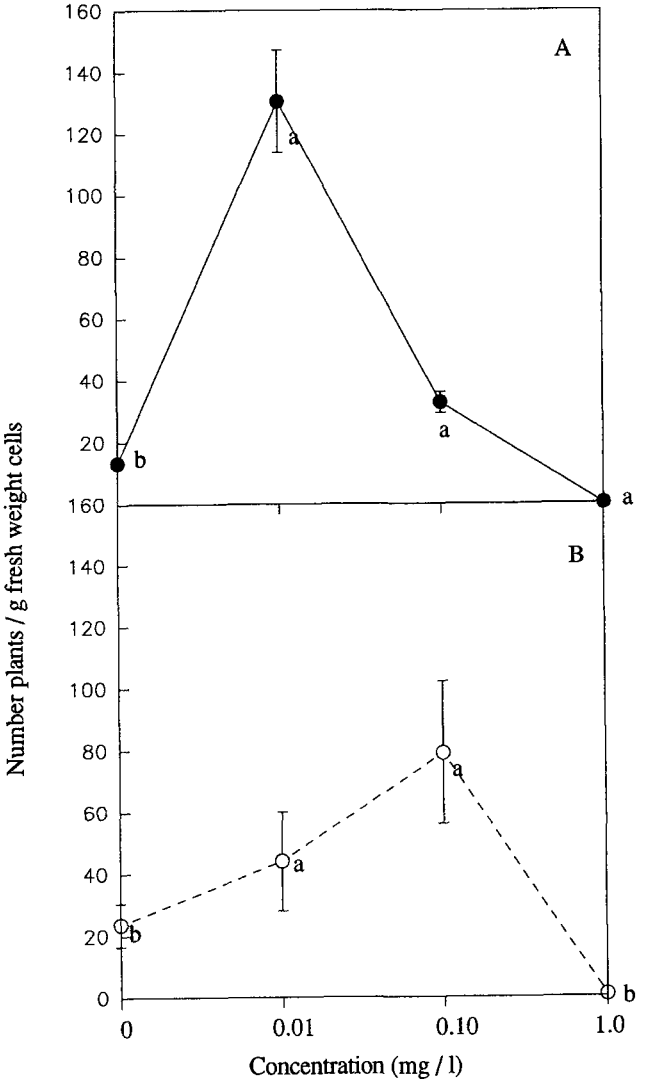

FIG. 2. Regeneration of plants from 9-mo.-old callus of 'Jenny Lee' cultured on regeneration medium supplemented with various concentrations of bialaphos (*A*) or PPT (*B*). *Standard error bars* shown. Means with different letters are significantly different at (*A*) $P \leq 0.05$ and (*B*) $P \leq 0.05$ according to Dunnett's test.

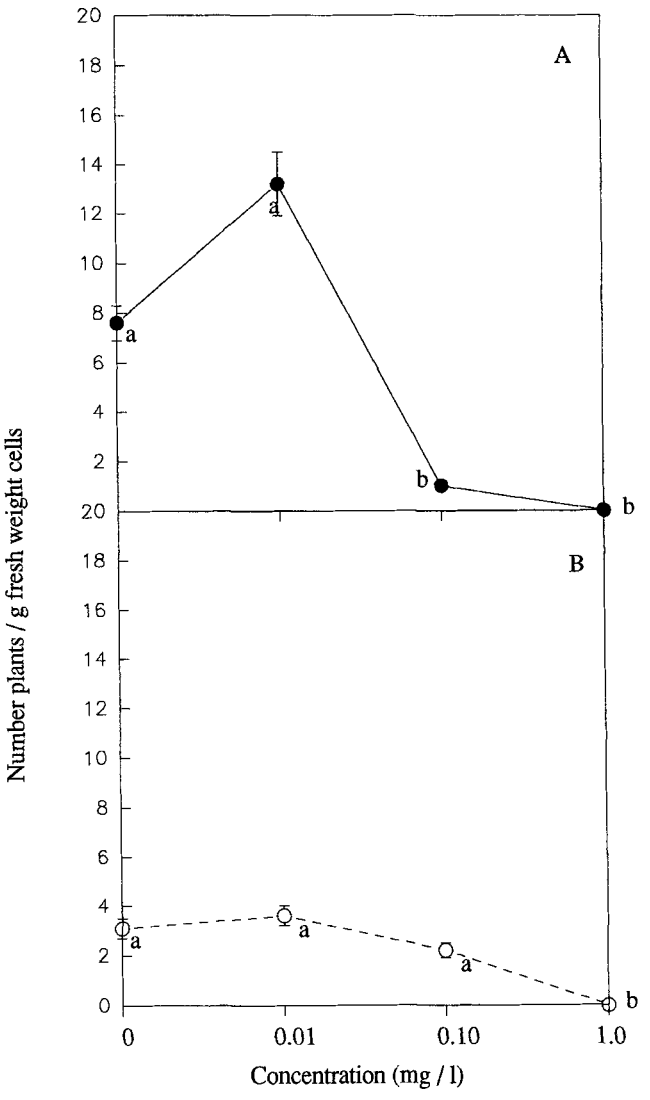

FIG. 3. Regeneration of plants from 4-mo.-old callus of 'Florida Flame' cultured on regeneration medium supplemented with various concentrations of bialaphos (*A*) or PPT (*B*). *Standard error bars* shown. Means with different letters are significantly different at $P \leq 0.05$ according to (*A*) Dunnett's and (*B*) the Tukey tests, respectively.

concentrations of PPT up to 1.0 mg/l. It appeared that 9-mo.-old cells were more responsive to bialaphos and PPT than 8-yr-old cells because the younger cells were stimulated to regenerate plants by either a lower concentration of bialaphos or PPT as compared to the 8-yr-old cells.

'Florida Flame' is a commercially important cultivar; it is more difficult to regenerate plants from 'Florida Flame' callus as compared to 'Jenny Lee' and other cultivars. The regeneration of 'Florida Flame' plants with bialaphos (0.01 mg/l)-supplemented medium was stimulated 1.7 times that of plants grown on regeneration medium lacking bialaphos (Fig. 3 *A*). Embryogenic callus of 'Florida Flame' was observed infrequently, and it was similar in appearance to that of 'Jenny Lee' (Fig. 4 *B*). PPT did not stimulate regeneration of 'Florida Flame' callus, which was similar to the result with 8-yr-old callus of 'Jenny Lee.'

Bialaphos and PPT inhibit glutamine synthetase resulting in accumulation of free ammonia in the plant. The NH_4^+:NO_3^- and other organic nitrogen sources have been shown to affect regeneration of various plant species (Halperin and Wetherell, 1965; Reinert et al., 1967; Gamborg, 1970; Wetherell and Dougall, 1976; Stuart and Strickland, 1984; Olsen, 1987; Grimes and Hodges, 1990; Abu-Qaoud et al., 1991; Shetty and Asano, 1991; Mordhorst and Lorz,

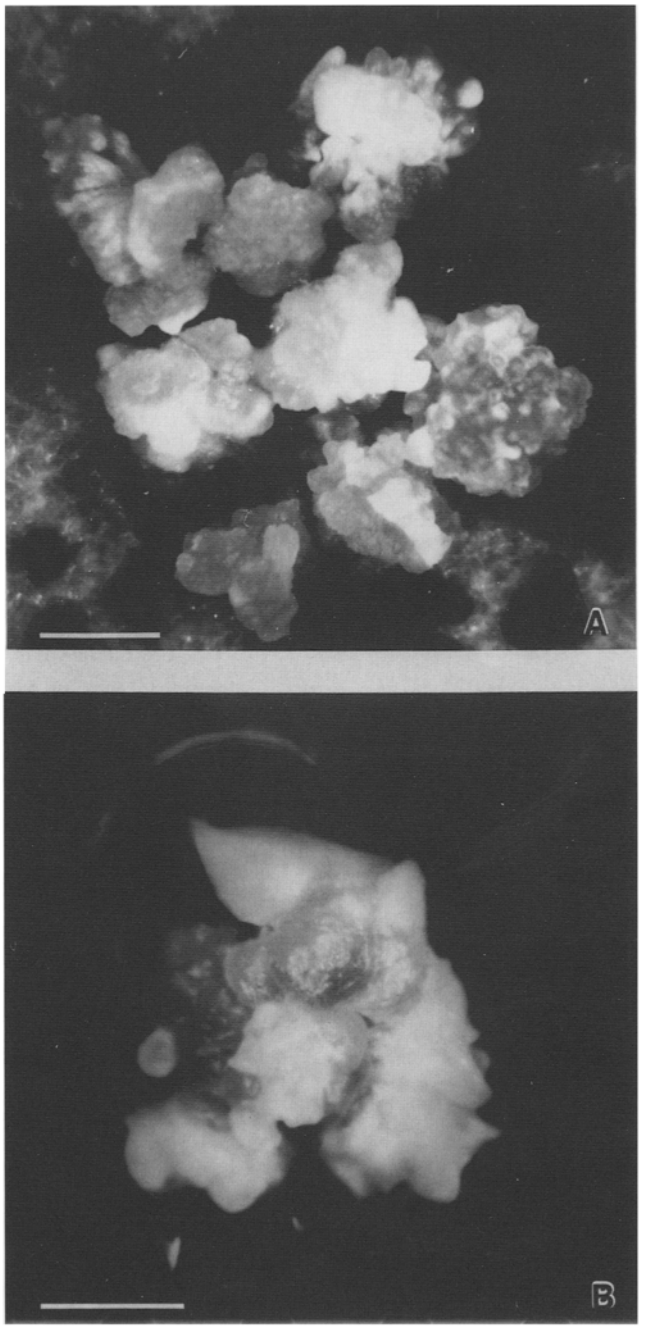

FIG. 4. *A*, 8-yr-old callus of 'Jenny Lee' cultured on regeneration medium supplemented with 0.10 mg/l bialaphos. *Magnification bar* = 5 mm. *B*, 4-mo.-old callus of 'Florida Flame' cultured on regeneration medium supplemented with 0.01 mg/l bialaphos. *Magnification bar* = 5 mm.

1993). This NH_4^+:NO_3^- ratio resulting from application of bialaphos and PPT may have affected regeneration of *Gladiolus* plants.

It is not known why bialaphos was more effective at stimulating regeneration of *Gladiolus* plants as compared to PPT. Bialaphos was also more effective than PPT when used as a selective agent to select for putative transformants of *Gladiolus*. Transgenic plants of *Gladiolus* were recovered after suspension or callus cells were cultured on either 1 mg/l bialaphos or 6 mg/l phosphinothricin, which were the concentrations determined to be required for death of nontransformed cells (Kamo et al., 1995). These results from both regeneration and selection experiments suggested that the tripeptide form of PPT may be more efficiently utilized by plant cells than unconjugated PPT.

In conclusion, bialaphos stimulated regeneration of *Gladiolus* plants from both long and short-term callus when included in regeneration medium at a concentration of either 0.01 or 0.10 mg/l, depending of the cultivar used.

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